# 1,25-Dihydroxyvitamin $D_3$ inhibits tenascin-C expression in mammary epithelial cells

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**Abstract Tenascin-C is an extracellular matrix protein with growth-, invasion- and angiogenesis-promoting activities. Tenascin-C is upregulated in breast carcinoma and stromal cells, and in many other cell types during tumorigenesis. We demonstrate that tenascin-C RNA expression is inhibited by 1,25-dihydroxyvitamin $D_3$ (1,25-$D_3$) in a variety of mouse and human mammary epithelial cell lines exhibiting normal or malignant phenotype. In EpH4 cells, the inhibition is maximum 24 h after 1,25-$D_3$ treatment and correlates with a dose-dependent reduction in the synthesis of tenascin-C protein. Furthermore, 1,25-$D_3$ also abolishes the induction of tenascin-C by serum or the tumor promoter 12-*O*-tetradecanoyl phorbol 13-acetate. The inhibition of tenascin-C expression may be relevant for the anticancer activity of 1,25-$D_3$.**



*Key words:* Tenascin-C; 1,25-Dihydroxyvitamin $D_3$; Mammary epithelial cell; Breast cancer

## 1. Introduction

1,25-Dihydroxyvitamin $D_3$ (1,25-$D_3$), the most active metabolite of vitamin D, has growth-inhibitory activities on cells from a series of cancers including breast, prostate, melanoma, sarcoma, pancreas, and renal cell carcinoma ([1–4] and references therein). In addition, 1,25-$D_3$ induces differentiation of leukemia cells [5]. In vivo, 1,25-$D_3$ also inhibits tumor growth in animal models [6,7]. 1,25-$D_3$ and several analogues have recently been shown to suppress DNA synthesis and the phosphorylation of retinoblastoma protein in breast cancer cells, and to promote active cell death via apoptosis [4,8]. Most (80–90%) primary human breast tumors express 1,25-$D_3$ receptors, a fact which seems to predict significantly longer disease-free intervals [2]. In a human trial, a vitamin D analogue has been shown to reduce by 50% the size of three out of 14 cutaneous breast cancer metastases [9]. In line with these data, epidemiological studies suggest a chemopreventive effect of vitamin D on the appearance of several types of cancer such as breast, prostate, and colon [10,11].

Tenascin-C is the prototype of the tenascin family of extracellular matrix glycoproteins thought to exert multiple effects on cell morphology, differentiation and physiology [12]. Tenascin-C is expressed at high levels during development, but in adult life it is expressed only during specific processes such as wound healing, inflammation and tumorigenesis. Activities of tenascin-C include growth promotion, anti-adhesive effects favoring cell motility, immunosuppression, and induction of angiogenesis. Both epithelial and stromal cells express tenascin-C in the mammary gland and breast tumors [13]. Tenascin-C expression seems to be inversely correlated with the differentiation of epithelial cells [14]. In fact, tenascin-C has been found to inhibit extracellular matrix/prolactin-dependent expression of the β-casein gene [15]. A correlation has been reported between high levels of tenascin-C expression and the malignancy of a series of human tumors, including breast and several other carcinomas [12,16,17]. Tenascin-C expression has recently been found to indicate a poor prognosis in human breast cancers [18]. Very importantly, tenascin-C expression in the area of the invasion border has been reported to be a strong predictor of distant breast cancer metastasis [19].

Based on the effects of 1,25-$D_3$ and on the increasing relevance proposed for tenascin-C in breast cancer, we have examined whether 1,25-$D_3$ may regulate tenascin-C expression in several normal and malignant mammary epithelial cell lines.

## 2. Materials and methods

### 2.1. Materials

1,25-$D_3$ was a kind gift from Productos Roche (Madrid, Spain). 12-*O*-Tetradecanoyl phorbol 13-acetate (TPA), estradiol, and bovine serum albumin (BSA) fraction V were from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and RPMI medium, fetal calf serum (FCS) and glutamine were all from Life Technologies (Paisley, UK). Autoradiography films, Amplify, and Promix used for protein labeling were from Amersham (London, UK). Protein A-Sepharose was purchased to Pharmacia (Sweden), and the rabbit polyclonal anti-human tenascin-C antibody used in the immunoprecipitation assays was also from Life Technologies.

### 2.2. Cell culture

Previously described EpH4 cells [20], and MDA-MB-231 and BT474 (American Type Culture Collection) mammary epithelial cells were grown and maintained in DMEM supplemented with 10% FCS and 1 mM glutamine. HC11 cells (a gift from Dr. N.E. Hynes, Friedrich Mieschner Institute, Switzerland) were grown in RPMI medium with the same supplements. When indicated, serum was omitted and replaced by 0.1 mg/ml BSA fraction V.

### 2.3. RNA preparation and Northern analysis

Purification of poly(A)$^+$ RNA, Northern blotting and hybridizations were performed as described [21]. Filters were washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate, pH 7.2 at 65°C. Membranes were exposed to Hyperfilm MP films. Densitometric analysis was performed in a La Cie scanner connected to a Macintosh IIci computer using Adobe Photoshop 2.0 and NIH Image Programs. Tenascin-C probe was a 1540 bp fragment (positions 1532–3072) from the mouse cDNA generously donated by Dr. P. Ekblom (Uppsala University, Sweden). SGP-2/TRPM-2 probe was the full-

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*Abbreviations:* BSA, bovine serum albumin; 1,25-$D_3$, 1α,25-dihydroxycholecalciferol (1,25-dihydroxyvitamin $D_3$); DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate


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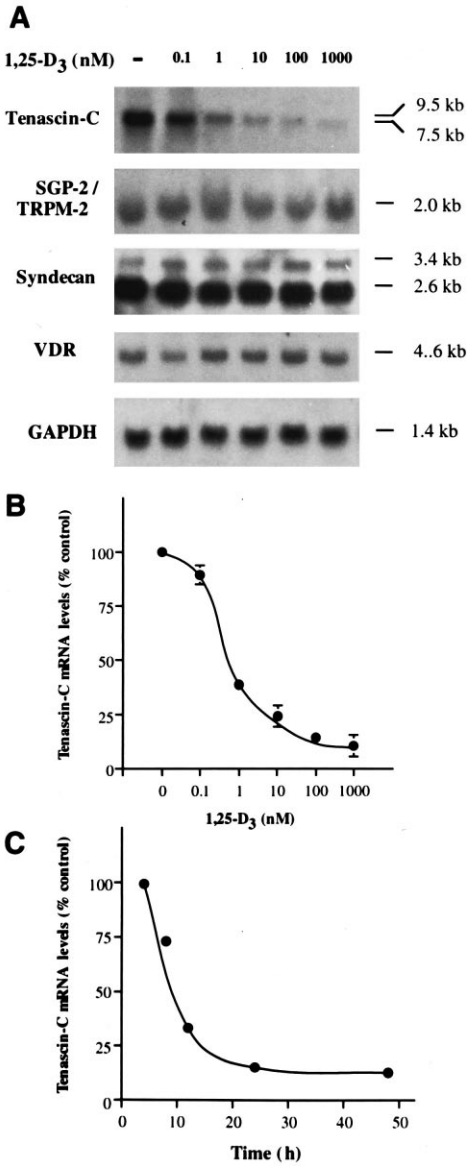


Fig. 1. Inhibition of tenascin-C gene expression by 1,25-$D_3$ in EpH4 cells. A: Dose-dependent regulation of tenascin-C gene expression in 1,25-$D_3$-treated EpH4 cells. Exponentially growing EpH4 cells were changed to serum-free medium and treated with the indicated concentrations of 1,25-$D_3$ for 24 h. Poly(A)$^+$ RNA (10 μg/lane) was analyzed by Northern blot using specific probes described in Section 2. Sizes of corresponding bands are indicated. B: Quantitation of the inhibition of tenascin-C gene expression by 1,25-$D_3$. Relative values of tenascin-C/GAPDH RNA levels with respect to that of untreated cells were calculated. Mean values corresponding to three independent experiments are shown. C: Kinetics of inhibition of tenascin-C gene expression by 1,25-$D_3$ (100 nM) in serum-free medium. Quantitation of relative values of tenascin-C/GAPDH RNA levels in 1,25-$D_3$-treated cells with respect to those of their corresponding untreated controls as calculated from Northern blots. Mean values of two independent experiments are shown.

length 1857 bp cDNA donated by Drs. M. Griswold and S.R. Sylvester (Washington State University, Seattle, WA, USA). A 1.1 kb *Bam*HI fragment of the pUC19-hsyn4 plasmid encoding the human syndecan cDNA, donated by Drs. M. Mali and M. Jalkanen (University of Turku, Finland), and a 1.1 kb *Eco*RI-*Sma*I of the pSG5-hVitDR+ encoding the human vitamin D receptor cDNA, donated by Dr. M. Zenke (Max-Delbrück Center, Berlin, Germany), were used.

*2.4. Immunoprecipitation assays*

Cells were grown to near confluence in 60 mm diameter dishes and treated or not with 1,25-$D_3$ for 24 h. After washing with phosphate-buffered saline, cells were incubated for 30 min in methionine- and cysteine-free, serum-free DMEM. They were then labeled for 3 h in the same medium containing 0.1 mCi/ml Promix. 1,25-$D_3$ was kept in the medium during the labeling period. After labeling, cells were collected in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) and centrifuged at 200×*g* for 10 min at 4°C. Supernatants were incubated with rabbit preimmune serum. Tenascin-C was immunoprecipitated by incubation with an appropriate rabbit polyclonal anti-human tenascin-C antibody for 3 h at 4°C. Immunoprecipitates were collected by incubation with protein A-Sepharose for 30 min at 4°C, washed four times with RIPA buffer and analyzed by electrophoresis in 6% polyacrylamide SDS gels followed by fluorography using Amplify.

## 3. Results

*3.1. Effects of 1,25-$D_3$ on tenascin-C RNA levels in EpH4 cells*

First, we analyzed the effects of 1,25-$D_3$ on tenascin-C RNA expression in EpH4 cells, a non-tumorigenic mouse mammary cell line with normal epithelial phenotype [20,21]. Treatment for 24 h with 1,25-$D_3$ caused a dose-dependent reduction in tenascin-C RNA levels (a major band of 9.5 kb and a minor band of 7.5 kb) in cells cultured in serum-free medium (Fig. 1A,B). Doses as low as 1 nM 1,25-$D_3$ resulted in a reduction of tenascin-C RNA content greater than 2-fold, while higher doses (10–1000 nM) led to a 6–8-fold reduction. These doses of 1,25-$D_3$ did not significantly inhibit DNA synthesis nor cause cell death during the assay (not shown). Further suggesting that the inhibition of tenascin-C is unrelated to effects on cell growth, we have observed that TGF-β causes an increase in tenascin-C expression while displaying a potent antimitogenic action on EpH4 cells (not shown). Also in line with this, 1,25-$D_3$ did not change the expression of SGP-2/TRPM-2, a gene induced in a number of cell lines undergoing apoptosis which has been reported to be induced by vitamin D analogues [8] (Fig. 1A). Likewise, neither the RNA level of the matrix proteoglycan syndecan nor that of the vitamin D receptor (VDR) is affected by 1,25-$D_3$ (Fig. 1A). Next, we examined the kinetics of the inhibitory action of 1,25-$D_3$. A reduction in tenascin-C RNA levels was already observed 8 h after beginning the treatment, and was maximum 16 h later (Fig. 1C). Since 1,25-$D_3$ did not change the low transcriptional rate of tenascin-C gene in run-on assays (not shown), it is highly probable that the inhibitory effect of 1,25-$D_3$ is a consequence of an effect on the stability of tenascin-C mRNA.

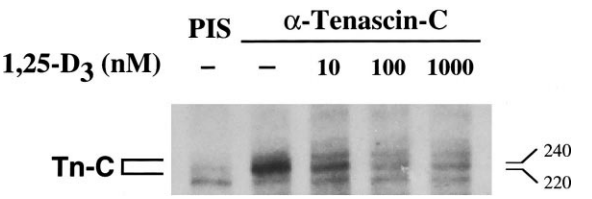


Fig. 2. Down-regulation of tenascin-C protein by 1,25-$D_3$ in EpH4 cells. Immunoprecipitation of tenascin-C polypeptides expressed in metabolically labeled EpH4 cells treated or not with the indicated concentrations of 1,25-$D_3$ for 24 h. The two tenascin-C bands ($M_r$ 240 000 and 220 000) are indicated. Preimmune serum (PIS) was used as control. Three separate experiments gave the same results.

### 3.2. Effects of 1,25-$D_3$ on tenascin-C protein synthesis in EpH4 cells

We used specific anti-tenascin-C antibodies to investigate whether the reduction in RNA levels correlated with an inhibition in tenascin-C protein synthesis. Proteins labeled in vivo during a 3 h pulse were immunoprecipitated from cells treated for 24 h with increasing doses of 1,25-$D_3$ or left untreated (Fig. 2). Two bands of $M_r$ 240 000 and 220 000 corresponding to tenascin-C polypeptides were detected. Low doses (10 nM) of 1,25-$D_3$ caused an overall (both bands) inhibition of tenascin-C protein synthesis of 40%, while at high doses (100–1000 nM) the inhibition was 65%. These results confirmed that 1,25-$D_3$ causes a reduction in tenascin-C expression in EpH4 cells.

### 3.3. Inhibition of tenascin-C expression by 1,25-$D_3$ in other mammary epithelial cell lines

Next, we extended the study to other mammary epithelial cell lines. In at least three additional lines of human (MDA-MB-231, BT-474) or mouse (HC11) origin which display different phenotypes and express variable levels of tenascin-C, 1,25-$D_3$ also exerted an inhibitory action reducing tenascin-C RNA levels in a range from 1.9- to 4.4-fold (Fig. 3). We also studied other cell lines such as MCF-7, T47D and ZR-75 which in agreement with previous reports did not show detectable tenascin-C gene expression ([13], and not shown).

### 3.4. Effect of 1,25-$D_3$ on the inducing activity of serum or tumor promoters on tenascin-C expression

To further investigate the inhibitory action of 1,25-$D_3$ on tenascin-C expression, we analyzed whether 1,25-$D_3$ could block the induction caused either by serum or by the tumor promoter TPA. To this purpose, EpH4 cells were first incubated in serum-free medium for 16 h in the presence or absence of 100 nM 1,25-$D_3$ and then treated for an additional 6 h with either 10% fetal calf serum or 100 nM TPA, alone or in combination with the same concentrations of 1,25-$D_3$. Control cells were left in serum-free medium with no additions. To assess the specificity of 1,25-$D_3$ action, estradiol was used in parallel. As shown in Fig. 4, 1,25-$D_3$ efficiently blunted the induction of tenascin-C expression caused by either serum or TPA. In contrast, estradiol had no effect.

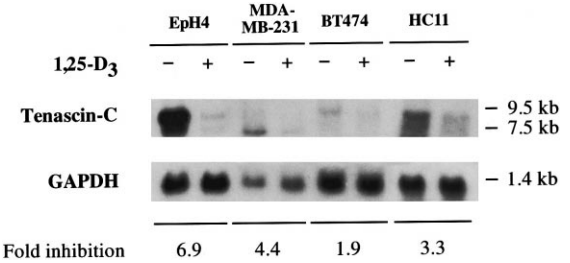


Fig. 3. Inhibition of tenascin-C mRNA levels by 1,25-$D_3$ in a variety of mammary epithelial cell lines. Poly(A)$^+$ RNA (10 μg/lane) extracted from the indicated cell lines treated or not with 1,25-$D_3$ (100 nM) for 24 h in serum-free medium was hybridized to specific probes. Sizes of the corresponding bands are indicated. Lower numbers represent the fold inhibition in tenascin-C mRNA levels caused by 1,25-$D_3$ treatment after normalization versus GAPDH mRNA levels.

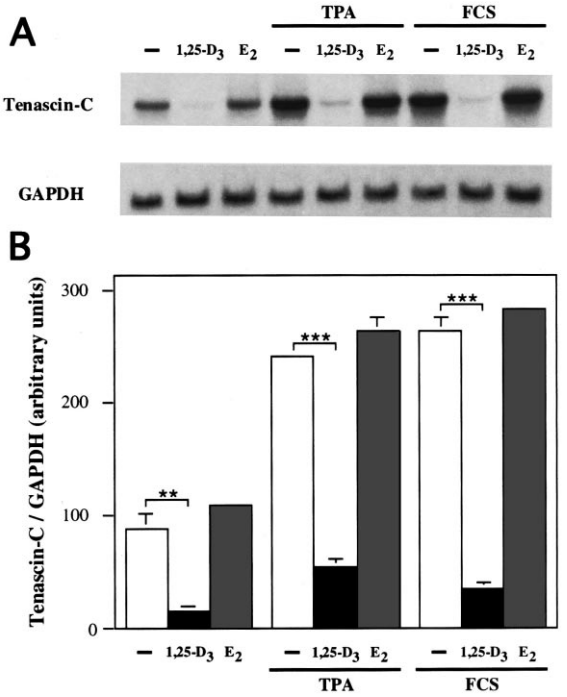


Fig. 4. 1,25-$D_3$ inhibits the increase of tenascin-C mRNA levels by TPA or FCS. A: EpH4 cells were treated with 1,25-$D_3$ (100 nM), estradiol ($E_2$, 100 nM) or left untreated for 16 h in serum-free medium. As indicated, TPA (100 nM) or FCS (10%) was then added for an additional 6 h. Poly(A)$^+$ RNA (10 μg/lane) was analyzed by Northern blot. B: Quantitation of the interference of 1,25-$D_3$ with the inducing activity of TPA and FCS on tenascin-C expression. Statistical analysis of the data obtained in three independent experiments; mean ± S.D. Statistical significance: $^{**}P < 0.01$; $^{***}P < 0.001$.

## 4. Discussion

In this study we report the inhibition by 1,25-$D_3$ of tenascin-C expression in a series of mammary epithelial cell lines of mouse and human origin. Interestingly, this action has been found in normal, polarized non-tumorigenic cells (EpH4) as well as in poorly polarized (HC11), in invasive tumorigenic (BT-474), and also in malignant dedifferentiated (MDA-MB-231) cells. Previously, the molecular basis for the anticancer activity of 1,25-$D_3$ was proposed to rely on a growth-inhibitory action, probably due to the blockade of the cell cycle by modulating the level of phosphorylation of the retinoblastoma protein, and on a pro-apoptotic activity [4,8]. Here, we demonstrate that 1,25-$D_3$ causes a rapid inhibition of tenascin-C expression which is independent of any effect on cell proliferation or survival. Supporting this, TGF-β induces tenascin-C in EpH4 cells which is a potent antiproliferative agent in these cells. Given the relevant roles proposed for tenascin-C promoting cell invasion and angiogenesis, and as an immunosuppressor [22], this finding shows that the antitumor activity of 1,25-$D_3$ is more complex than expected, and emphasizes the rationale of its use as a chemopreventive agent in cancer.

In spite of the proposed role of tenascin-C in mammary gland morphogenesis, physiology, and tumorigenesis, very little is known about its regulation in mammary epithelial cells. In HC11 cells tenascin-C has been found to be up-regulated by TGF-β and down-regulated by epidermal growth factor [14]. In addition, a down-regulation of tenascin-C expression

by antiprogestins has been reported in rat mammary tumors [23]. Studies carried out with different cell types such as fibroblasts, vascular smooth muscle, astrocytes or bone marrow stromal cells in which tenascin-C is regulated by different growth factors, cytokines, and steroids have shown that the regulation of tenascin-C expression is cell-specific [24]. The analysis of the promoter region of the chicken and human tenascin-C genes has not yet given much information about possible transcriptional regulators [24]. Recently, the dissection of the mouse tenascin-C promoter has revealed the existence of multiple elements for a series of transcription factors (Krox-24, Brn-2, NF-1, and POU factors) which function in a complex, cell-specific fashion [25].

Our results suggest a direct effect of 1,25-$D_3$ on tenascin-C gene expression which may play a role in the physiological regulation of the gene. This effect seems to be mostly at the level of mRNA stability. In addition, the blockade of the induction of tenascin-C by serum or TPA supports the idea that 1,25-$D_3$ may act also by inhibiting the AP-1-dependent expression of this gene.

Given the relevant activities proposed for tenascin-C in tumorigenesis, particularly in breast malignancies, the inhibitory effect described here most probably contributes to the antitumor activity of 1,25-$D_3$.

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